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FGFR3 expression in primary invasive bladder cancers and matched lymph node metastases

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Runninghead: FGFR3 in invasive bladder cancer and matched metastases

Key Words: Bladder cancer, FGFR3 protein, immunohistochemistry, metastasis, tissue microarray analysis

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ABSTRACT

Purpose: FGFR3 is considered a good therapeutic target in bladder cancer. However, it is not known whether the FGFR3 status of primary tumors is a surrogate for their related metastases, which must be targeted by FGFR-targeted systemic therapies. We aimed to assess FGFR3 protein expression in primary bladder tumors and matched nodal metastases.

Materials and Methods: Matched primary tumor and nodal metastasis from 150 bladder cancer patients clinically staged as N0M0 were examined. Four samples from each patient were incorporated into a tissue microarray and FGFR3 expression assessed by immunohistochemistry. FGFR3 expression was tested for association with categorical clinical data using Fisher's exact test, and overall OS and recurrence free survival RFS using Kaplan-Meier analysis.

Results and Conclusions: Duplicate spots from primary tumors and lymph node metastases showed high concordance (primary tumor: OR=8.6, $p<0.001$ metastases: OR=16.7, $p<0.001$. Overall, levels of FGFR protein expression did not differ between primary and metastatic lesions ($p=0.78$). Upregulated expression was recorded in 53 of evaluable 106 primary tumors spots and 56 of the matched metastases. Concordance between FGFR3 expression levels in matched primary tumor and metastasis (n=79) was high (OR=8.45, $p<0.001$). In 15 patients only the metastasis had upregulated expression and in 12 patients, only the primary tumor. OS and RFS were not related to FGFR3 expression.

FGFR3 expression in matched primary and metastasized bladder cancer showed good but not absolute concordance. We conclude that in the majority of patients FGFR3 status of the primary tumor can guide selection of FGFR-targeted therapy.

INTRODUCTION

Fibroblast growth factor receptor 3 (FGFR3) is commonly activated by mutation, over-expression or chromosomal translocation in UC of the bladder.[1-3] Up to 80% of superficial papillary tumors and 10-20% of muscle-invasive tumors contain activating point mutations. In advanced UC, the most common finding is upregulated expression of wildtype FGFR3 in 40-50% of cases.[2] FGFR3 is considered a good therapeutic target in UC. In UC cell lines, inhibition by knockdown, small molecules or antibodies has a profound effect including inhibition of xenograft growth.[4-11] Several clinical trials of FGFR-targeted agents are now planned or in progress in UC. The use of such inhibitors can be envisaged as part of neoadjuvant treatment or in the metastatic setting. Metastatic disease is the most common cause of death in bladder cancer but currently used chemotherapy regimes have limited efficacy and overall survival is low (10 year-survival for patients with lymph node positive disease $\underline{\sim}$ 30%).[12] Thus, there is an urgent need for improved approaches to therapy for these patients. FGFR inhibitors may be relevant agents to apply in combination with platinum-based chemotherapy.

Successful treatment of advanced disease must target metastatic lesions. It is not yet clear how closely the molecular profile of metastatic UC mirrors that in the primary tumor. As biopsies of metastases are not routinely available at the time of selection of therapy, it is important to assess whether the primary tumor can act as a surrogate. Few studies have examined UC metastases and to date none have examined FGF receptors. Therefore, we evaluated the frequency and prognostic impact of FGFR3 protein overexpression in a series of primary UC with matched lymph node metastases treated by radical cystectomy and extended pelvic lymphadenectomy.

MATERIALS AND METHODS

Patients and follow-up

The cohort comprised 150 bladder cancer patients without preoperative evidence of metastases (i.e. by physical examination, chest x-ray, intravenous urography, bone scan, and pelvic computed tomography, when available) but with lymph node metastases on pathologic examination (Table 1). All patients underwent extended pelvic lymphadenectomy with cystectomy as a single procedure between January 1985 and April 2008 at the Department of Urology, University of Bern. No neoadjuvant therapy was given. Postoperatively, the patients were followed prospectively according to a standard protocol with examinations at the Department of Urology or by urologists in private practice at 3 months and 6 months, then at 6-month intervals until 5 years, and then at yearly intervals thereafter.

Treatment and pathology

All patients were treated by cystectomy and bilateral extended pelvic lymphadenectomy according to standard protocols described previously.[13, 14] The surgical specimens were processed as described previously.[14] Tumors were staged according to the 7th Union Internationale Contre le Cancer classification.[15]

Construction of the tissue microarray

The tissue microarray, which has been used in several previous studies[16-18], was constructed in the Institute of Pathology, University of Bern with six tissue cores (0.6-

mm diameter) per patient: two each from normal urothelium, primary tumor (centre and invasion front), and a nodal metastasis.

Immunohistochemistry

For detection of FGFR3 protein, deparaffinized and rehydrated sections were treated with 3% hydrogen peroxide (Sigma, Poole, UK), microwaved for 20 minutes, and blocked with an Avidin Biotin blocking kit (Vector Laboratories, Peterborough, UK). Primary antibody (FGFR3 B9, 1:100, Santa Cruz, CA, USA) was applied for 1 hour at room temperature and detected with a biotinylated secondary antibody and 3,3-diaminobenzidine. Slides were counterstained with Mayer's haematoxylin, dehydrated and mounted.

Immunostaining was assessed by three independent observers (PH, MK, RT), who were blinded to all clinical information. A semi-quantitative scoring system was adopted: 0, all tumor cells negative; 1, faint but detectable positivity in some or all cells; 2, weak but extensive positivity; 3, strong positivity (regardless of extent). For the statistical analysis, tumors scoring 0 and 1 were grouped as low (L) and tumors scoring 2 and 3 as high (H) (Figure 1). When two cores from the same patient where discordant the highest score was used for analysis.

Statistical analyses

FGFR3 expression was tested for association with categorical clinical data using Fisher's exact test, and OS and RF) using Kaplan-Meier analysis. Significance of predictors in survival analysis was assessed using the log-rank test for univariate analysis. For the

analysis of metastatic tumor phenotype the ratios have been calculated for total and lower metastases' diameter, and total number of metastases. All statistical calculations were carried out with SPSS for Windows version 20.

RESULTS

FGFR3 protein expression in primary tumors and metastases

FGFR3 protein was not detected by immunohistochemistry in the normal urothelial samples. In the tumor tissues, FGFR3 protein was detected in cytoplasm and at the cell membrane. Variable levels of expression were noted. Initially, we assessed concordance between tissue cores from the same tumor component. Cores from the centre of primary tumors were evaluable in 123 cases. Of these, 55 (44.7%) had score H and 68 (55.3%) had score L. Cores from the invasion front of primary tumors were evaluable in 110 cases, 34 (30.9%) with a score H and 76 (69.1%) with score L. Of these, 97 cases had paired centre and invasion front cores available for analysis. Seventy-three (74.5%) of them were concordant (OR=8.6, p=0.000003, Fisher's exact test) (Table 2). At least one core of metastatic tissue from 102 patients were evaluable. Forty-eight of these (47.1%) had score H and 54 (52.9%) score L. The second core from 104 patients was evaluable, with 41 (39.4%) score H, and 63 (60.6%) score L. Both biopsies were available from 90 patients, 71 of which (78.9%) were concordant (OR=16.7, p=0.0000002, Fisher's exact test) (Table 2).

We then compared levels of expression in primary tumors and metastases. Overall, the levels of FGFR3 protein expression did not differ between primary tumors

and metastatic lesions (p=0.78, Mann-Whitney test). FGFR3 protein expression was assessable in 106 matched pairs of primary tumors and metastases. Fifty-three of the primary tumors and 56 of the metastases showed high level expression.

In 79 patients, there was concordance between FGFR3 expression level in their primary tumors and matched metastasis (OR=8.45, p=0.000007, Fisher's exact test). In 15 patients the primary tumor had low protein expression and the matched nodal metastasis had high expression. The converse was observed in 12 patients.

Relationship of FGFR3 status with metastatic phenotype

The relationship of upregulated FGFR3 expression to number and size of lymph node metastases was examined. The relationship of high versus low FGFR3 expression to number of positive lymph nodes relative to total number of nodes (median: 0.13 vs 0.21 p = 0.25, Mann-Whitney test) average nodal size (mean: 0.79 cm vs 1.04 cm; p = 0.065, t test) and maximum diameter of metastases (mean: 1.82 vs 1.55, p=0.30, t-test, or median = 1.65 vs 1.00, p=0.063, Mann-Whitney test) was examined. Whilst the number of positive nodes and the diameter of metastatic lesions appeared to be related to FGFR3 status (Figure 2), these differences were not statistically significant. Similarly, tumor stage and extracapsular extension of lymph node metastases were not associated with FGFR3 status.

Relationship of FGFR3 status to survival

In univariate analyses, OS did not differ significantly for patients with FGFR3 overexpression in the primary tumors compared with those patients without (p=0.68) (Supplementary Figure 1). This was also the case for patients with FGFR3

overexpression in metastases (p = 0.85) (Supplementary Figure 1). Similarly, FGFR3 expression status in either primary tumors or metastases did not correlate with RFS (p=0.70 and 0.94 respectively) (Supplementary Figure 2).

In multivariate analyses, advanced primary tumor stage (pT3/4 vs pT1/2; p = 0.019) and extracapsular extension of lymph node metastases (p = 0.001) were the only independent adverse risk factors for OS. FGFR3 overexpression in primary tumors (p=0.66) and metastases (p=0.88) failed to add independent prognostic information.

FGFR3 status and adjuvant chemotherapy

A recent study reported that high levels of FGFR3 expression were related to adverse outcome in invasive UC treated with adjuvant chemotherapy [19]. In this series of cases, 63 patients had received adjuvant chemotherapy. In 37 cases, this was cisplatin-based. We examined the relationships of FGFR3 expression in primary and metastastic tissue and chemotherapy (no chemotherapy, any chemotherapy and cisplatin-based chemotherapy) with overall and recurrence-free survival. No significant relationships were found (Supplementary Figure 3).

DISCUSSION

Selection of patients for targeted therapies requires knowledge of the presence of the relevant protein target. In non-invasive bladder tumors the presence of FGFR3 mutation shows strong correlation with high protein expression levels [2], but this relationship is not so clear for advanced bladder cancer. [19] Thus measurement of protein levels rather than mutation status appears most relevant in this group of patients. Our

finding of expression in 45% of primary tumor tissues is in accord with previous studies, which have reported that 40-50% of muscle invasive tumors show upregulated expression.[2, 9, 19]

Ideally, patient selection for systemic treatment requires knowledge of the status of a therapeutic target in distant tumor metastases. In bladder cancer the only tissues routinely available are primary tumor and/or local recurrences. If these tissues can provide a surrogate for disseminated tumor cells, then treatment may be selected with confidence. Encouragingly, we have found a similar frequency of FGFR3 expression in both primary tumors and metastatic samples ($p=0.78$), confirming that a significant proportion of patients may benefit from FGFR-targeted approaches to therapy. Importantly, there was good correlation between FGFR3 expression in primary tumors and their paired metastases with concordant scores in 74.5% of patients. Fourteen percent of samples expressed FGFR3 in the nodal metastasis but not in the primary tumor, which if translated into a clinical trial setting might lead to relevant treatment being withheld in these cases. Conversely, 11% of patients in whom expression was recorded in the primary tumor but not metastasis might be over-treated. This level of precision in selection of patients may be acceptable when considered in light of the current absence of predictive biomarkers for chemotherapy response and the considerable morbidity suffered by patients in whom no tumor response is recorded.

The basis for the discordant results is not clear. Possibilities include dissemination of cells that represent only a minor sub-population in the primary tumor and effects of the tissue microenvironment on gene expression at the metastatic site. In this study, only two tissue cores were analysed for each tumor component. Therefore,

sampling differences may be a limitation that would not allow heterogeneity in the primary tumor to be recognised. Assessment of whole tissue sections of the discordant samples may clarify this.

The role of FGFR inhibitors in the treatment of advanced bladder cancer is not yet clear and awaits the results of early clinical trials. Interestingly, a recent study of the relationship of FGFR3 mutation and expression status to outcome in muscle-invasive bladder cancer treated by cystectomy with or without adjuvant cisplatin-gemcitabine chemotherapy, reported FGFR3 over-expression to be an independent predictor of reduced OS and RFS. [19] The present study did not reveal the same relationship. The reason for this is unclear, but this potential relationship should be examined in a much larger series of patients to clarify the situation as such a relationship could have major predictive application.

The finding of good concordance between FGFR3 expression in these paired samples does not indicate that all molecular features are likely to be similar. For example, the use of expression or DNA-based markers for the same gene may not give the same results. Thus detection of ERBB2 amplification by fluorescence *in situ* hybridization showed high concordance whilst ERBB2 protein expression showed lower concordance in the tissue samples used here[18]. Unlike expression changes, a genomic alteration is unlikely to be lost from cells following engraftment at a metastatic site, and intra-tumor heterogeneity appears a more likely explanation in this case. More detailed examination of both FGFR3 and ERBB2 in the gross specimens of these tissues will be of great interest.

Our study has some limitations. Despite including a relatively large number of

matched samples the study might be underpowered to show statistically significant differences. For example, number of positive nodes and diameter of the metastatic deposits both appeared to show a relationship to FGFR3 status but neither were statistically significant. These features merit investigation in a larger sample series. **In this study, we were unable to assess FGFR3 mutation status. Whilst protein levels may be the most relevant measure in invasive bladder tumors, it will be important to confirm this by assessment of both genomic alterations and expression changes in any future studies. The use of a more robust measure of protein expression level, for example by image analysis, may also be included.**

The arguments for using IHC to assess therapeutic targets are strong. Targeted therapy works at the protein level and protein expression is not always directly related to genomic features such as mutation or gene amplification. However, one of the limitations of IHC is its variability in reporting. This often reflects differences in antibodies, detection kits, protocols and methods of interpretation. Here we simplified our scoring criteria by grouping samples into only two groups (low and high). Assessment of a therapeutic target has different requirements than assessment of a prognostic indicator. Thus, scoring based on the worst feature or presence of minor cell population in a tumor may be inappropriate for application in clinical practise and new systems may be required. Our study provides encouragement to now assess gross samples from a similar cohort of patients.

CONCLUSIONS

The expression status of FGFR3 protein in lymph node metastases removed at

cystectomy shows good concordance with the expression status of the related primary tumor. We conclude that in the majority of patients, FGFR3 status in primary tumor tissues provides a good surrogate for status of metastatic disease and that therapeutic decisions can be based on this.

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Figure Legends

Figure 1. Detection of FGFR3 by immunohistochemistry. A. Strong FGFR3 expression (score 3). B. Weak but extensive positivity (score 2). C. Faint but detectable positivity (score 1). D. Absence of staining (score 0). Size bar = 100μm.

Figure 2. A. Relationship of FGFR3 expression to number of positive lymph nodes (median: 0.13 vs 0.21; p = 0.31). Y- axis: fraction of lymph nodes with metastasis; X – axis: FGFR3 expression (low or high). B. Relationship of FGFR3 expression to diameter of metastatic deposit (median: 0.79 cm vs 1.04 cm; p = 0.065). Y- axis: mean metastasis diameter in cm; X – axis: FGFR3 expression (low or high).